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CHARACTERIZATION OF THE INTEGRATED VIRUS DETECTION SYSTEM (IVDS) USING MS-2 BACTERIOPHAGE

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The detection and analysis of viruses have been goals of science for more than 70 years, following the first real evidence that a new type of microorganism was responsible for diseases in man and animals. These new microbes were smaller than bacteria, which have now been well documented, classified, and studied. Their small size made classifying the new microbes more difficult, and the field of virology has been advanced by biochemical techniques rather than by direct examination. Advancements in electron microscopy in more recent times have made advances in this area, and much has been reported on the physical features of more than 21 virus families. All these historic techniques are time consuming and require special knowledge and specialized chemicals and preparations to be successful. Capitalizing on the physical characteristics, it was possible to separate the families and count the individual viruses in a new and dramatic way, using easily obtained materials and simple-to-operate techniques. The only materials used in the Integrated Virus Detection System (IVDS) are a buffer, CO_2 gas, and butanol. The work in this report presents the advances in the concentration and counting phases of IVDS. Results indicate a practical, easy to use device, which can count viruses in near real time. The IVDS can, in its final configuration, be expected to analyze a sample, count the viruses present, and give a preliminary identification for all viruses.

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SUMMARY

This report documents the results of DARPA-funded work on the Integrated Virus Detection System (IVDS). Substantial advancement was achieved with the continued hardware development of the Gas-phase Electrophoretic Mobility Molecular Analyzer (GEMMA) by incorporating a number of modifications and upgrades to the GEMMA detector unit. Ongoing laboratory studies further validate that with continued funding, the IVDS is a feasible solution for providing near real-time, stand-alone monitoring for all viral threats, known or unknown.

Additional work has focused on the machining and integration of a new, second-generation, three module ultrafiltration (UF) stage, and testing the UF-GEMMA tandem ability to purify and detect viruses at various concentrations and levels of background impurities. A density-gradient ultracentrifugation stage, which has been purchased but not yet integrated into the IVDS, will accomplish a high degree of extraction and purification of viruses from high-background samples. Knowing this, the purification requirements of the UF and GEMMA stages will be greatly reduced once the centrifuge is integrated. A range of impurity levels were investigated in the ongoing studies (up to 0.5% cesium chloride and 1.0% thyroglobulin) in order to ensure that the worst possible cases were addressed.

The results of these tests illustrate:

- the 2nd and 3rd modules of the UF performed well for retention volumes down to 10 microliters
- the 1st module has excellent purification performance even for large proteins but may need modification to minimize loss of viruses
- improvements made on the GEMMA--which shows superb performance when vapor-lock is avoided--appear to be bringing us to operational consistency
- current sensitivity is 10^7 virus particles or between 10^3 and 10^7 plaque forming units (pfu), which can be readily improved to below 10^3 pfu (that of polymerase chain reaction or PCR) by the substitution of a new condensate particle counter (CPC) and new electrospray (ES) unit into the GEMMA

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PREFACE

The work described in this report was performed as part of Defense Advanced Research Projects Agency (DARPA). This work was started in March 1998 and completed in September 1998.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

Acknowledgments

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CHARACTERIZATION OF THE INTEGRATED VIRUS DETECTION SYSTEM (IVDS) USING MS-2 BACTERIOPHAGE

1. Introduction

The detection and analysis of viruses has been the goal of science for more than 70 years, following the first real evidence that a new type of microorganism was responsible for diseases in both man and animals. These new microbes were smaller than bacteria which have now been well documents, classified and studied. Their small size made classifying these new microbes more difficult and the field of virology has been advanced by biochemical techniques rather than by direct examination. Advancements in electronmicroscopy in more recent times have made advances in this area and much has been reported on the physical features of more than 21 virus families. All these historic techniques are time consuming, require special knowledge and specialized chemicals and preparations to be successful. Capitalizing on the physical characteristics it was found possible to separate the families and count the individual viruses in a new and dramatic way using easily obtained materials and simple to operate techniques. The only materials used in the Integrated Virus Detection System are a buffer, CO_2 gas, and butanol. The work in this report presents the advances in the concentration and counting phases of IVDS. Results indicate a practical, easy to use device, which can count virus in near-real-time. IVDS can, in its final configuration, be expected to analyze a sample, count the viruses present, and give a preliminary identification for all viruses.

Background

The IVDS project began more than six years ago with the development of the theory and concepts needed to build a device which could analyze virus particles which are among the smallest known microorganisms[1]. Biochemical techniques were examined and excluded because they are inherently limited in the number and types of virus and virus like particles they can analyze in a short time frame[2,3]. The physical characteristic methodology was found to be generic in nature, quick, and unlimited in its ability to count and analyze all viruses. Experiments to date have advanced this early work and now the system can routinely examine such viruses as MS-2, a regularly used simulant for viruses. Current findings report advances in the development of the concentration and counting stages.

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[1] Wick, C.H., Yeh, H.R., Carlon, H.R., and Anderson, D., *Virus Detection: Limits and Strategies*, ERDEC-TR-453, December 1997.

[2] Wick, C.H., Yeh, H.R., Carlon, H.R., and Anderson, D., *Biological Warfare: Inherent Limits of Schemes for the Detection of Airborne Viruses*, ERDEC-TR-465, January 1998.

[3] Wick, C.H., Yeh, H.R., Carlon, H.R., and Anderson, D., *Quasi-Realtime Monitor for Airborne Viruses*, ERDEC-TR-459, January 1998.

2. The Integrated Virus Detection System (IVDS)

The IVDS automates detection and monitoring of submicron particles or macromolecules, and in particular, viruses and virus-like materials in many fluids, including biological fluids, water, and air. The detection of viruses of any family, genus and species, not limited to a particular family of focus, is an extremely challenging problem for current technology. IVDS is a breakthrough in many areas providing an accurate identification and analysis (including sample concentration and purification) in less than 15 minutes.

The virus sample is purified and concentrated using an ultrafiltration (UF) process. The UF module, shown in Figure 1 and Figure 2, allows the removal of interfering proteins and other biological entities while retaining the virus species for further study. The UF module is described in detail in Section 2.1.

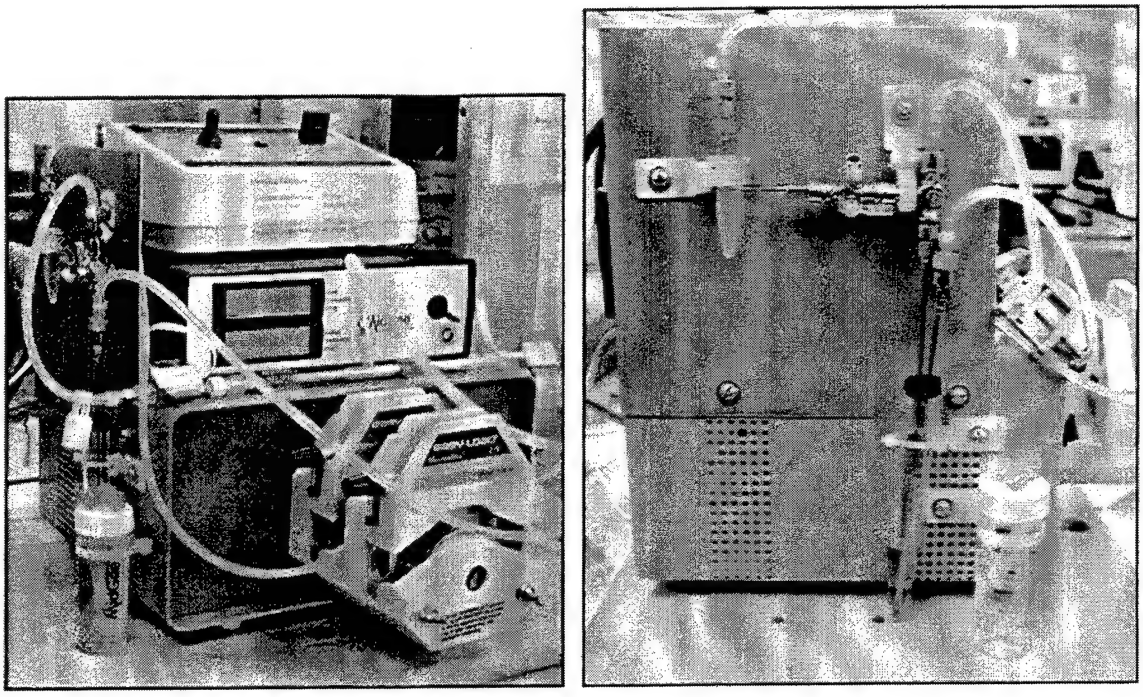

Figure 1 Front and Side View of UF Module 1

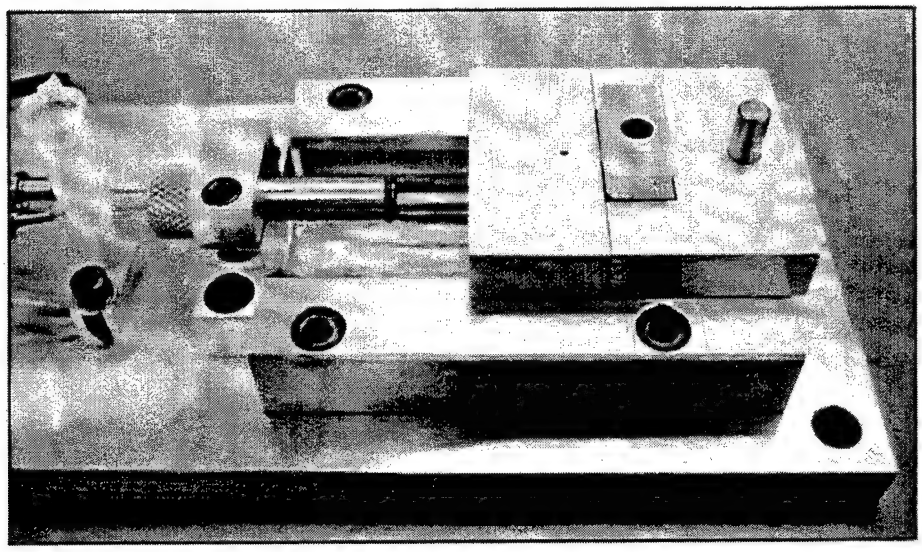

Figure 2 View of UF Module 2

The Counting Stage, or GEMMA unit, shown in Figure 3, uses an electrospray nozzle, differential mobility analyzer, and a condensation particle counter. The technologies are available commercially and configured individually or in combination. The Counting Stage is described in detail in Section 2.2.

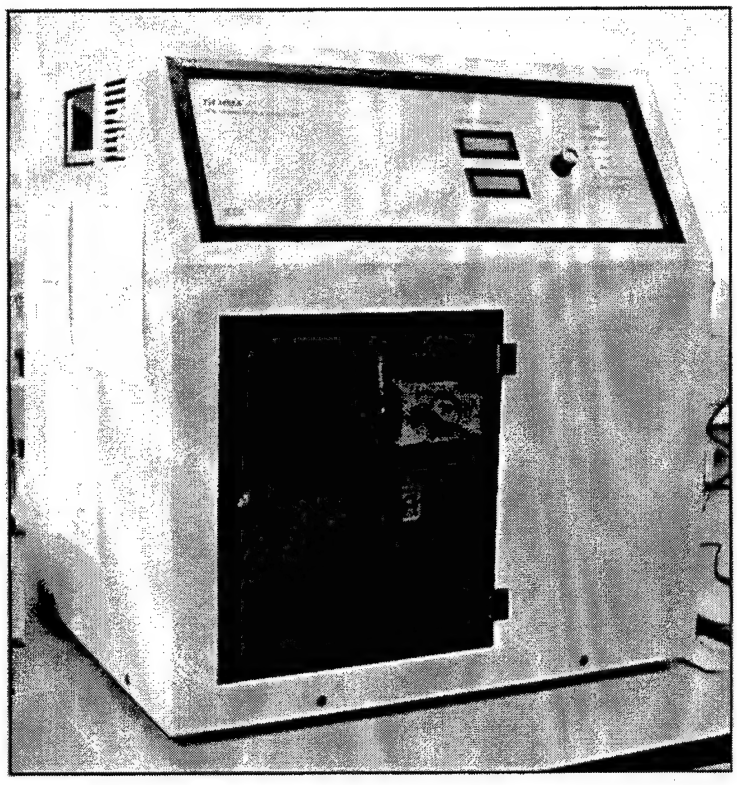

Figure 3 Front View of GEMMA Unit

The IVDS output can be tailored to a variety of PC-based reporting hardware. Computer outputs are described in greater detail in Section 2.3.

2.1 UF Module.

The first module of the three-module UF stage is a hollow fiber-based tangential flow filtration system. These filtration systems operate by pumping the feed stream through the hollow fiber, as shown in Figures 4 and 5. As the solution passes through the fiber, the sweeping action of the flow helps to prevent clogging of the fiber. A pressure differential forces the filtrate through the fiber, while the virus feed stream is purified and concentrated. There are available a wide range of pore sizes for the fibers. This filtration technique can reduce volumes from 5 ml to 0.2 ml. The first module has been completed and tested successfully, as reported in more detail in Section 5.6.6.

The second module, as shown in Figure 6, which is a flat-membrane diafiltration apparatus, and reduces volume from 0.2 ml to 400-10,000 nanoliters, has also been completed and tested successfully. To our knowledge, no UF device with a retention volume of this order of magnitude has even been built.

The third module, which accepts output from the second module and inputs it to the detector, has also been completed and tested successfully. The third module can also accept outputs from other sample feed streams that have been used or considered. For example, capillary sample holders would allow nanoliter-size samples to be transported by mail to a centrally operated GEMMA unit.

A key feature that has been built into the overall UF design is the independence of the three modules, which has both performance and practical implications. One performance implication is that the first module can, after passing its output to the second module, move to processing sample #2 while the second module is still processing #1, likewise with the second and third modules, thus reducing total operating time. It also makes for easier maintenance to replace one module with a spare and rapidly bring the IVDS back on line.

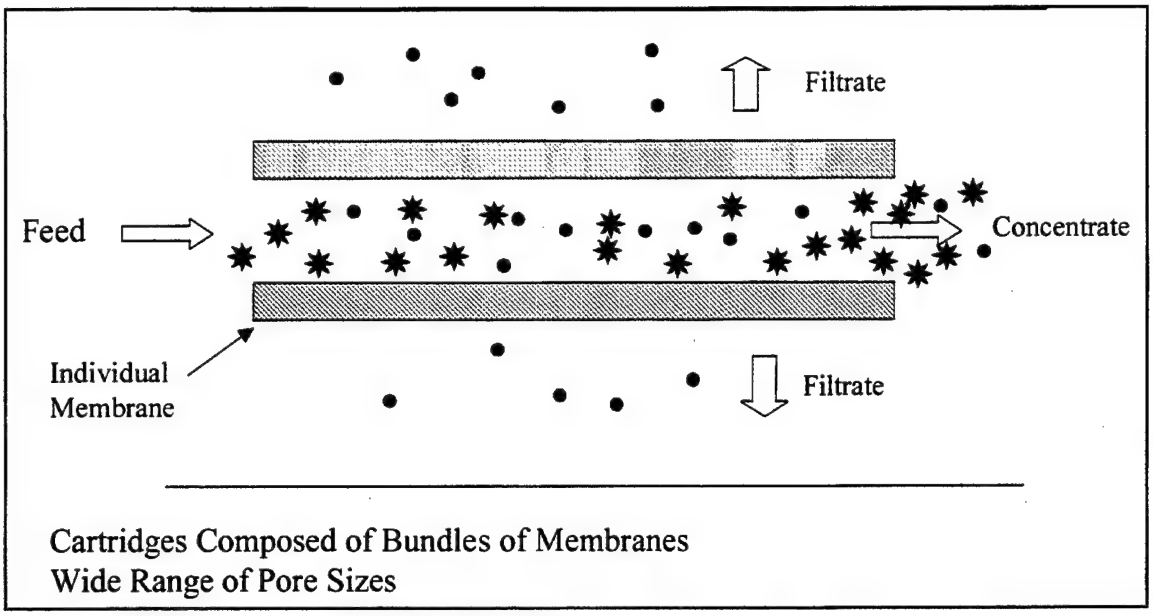

Figure 4 Cross Flow Filtration - Individual Fiber

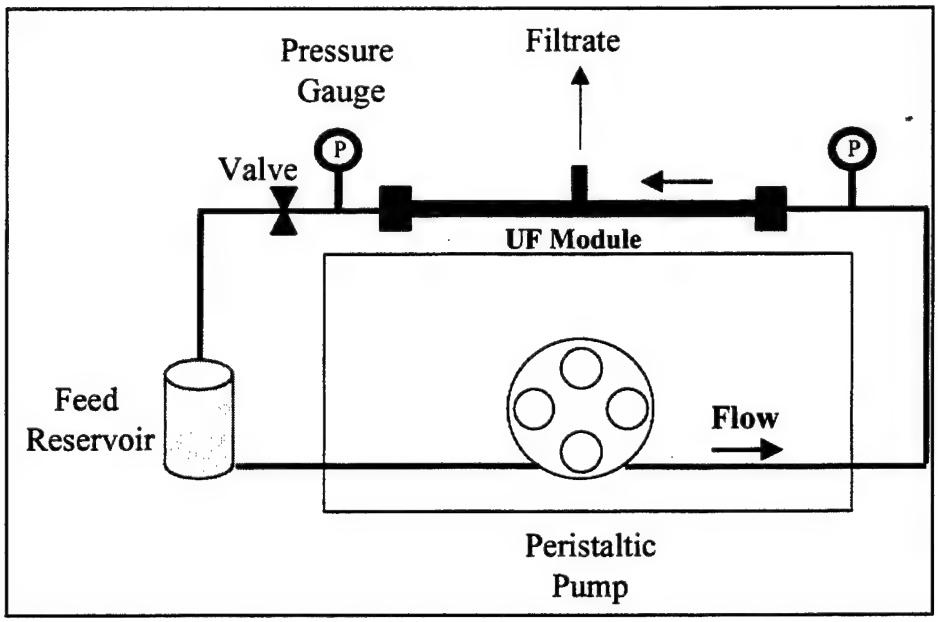

Figure 5 UF Module 1 Using the Cross Flow Filtration Process

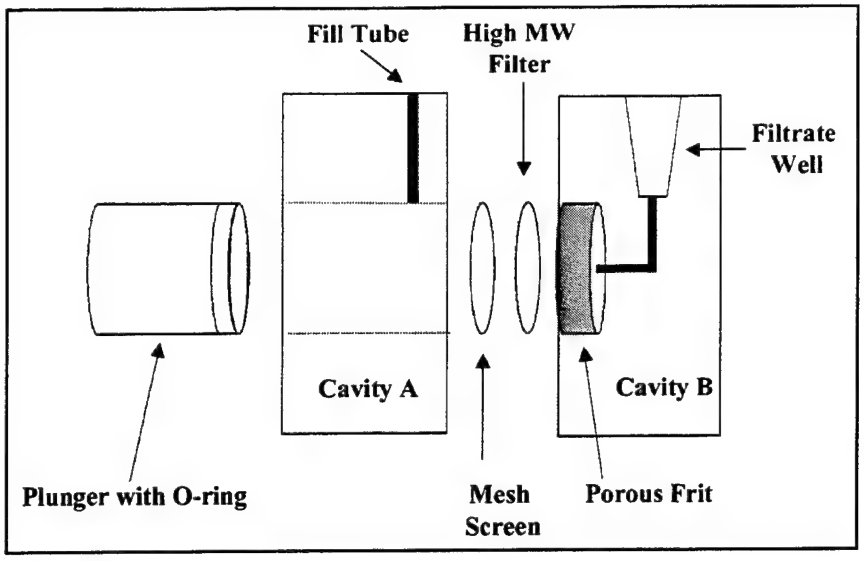

Figure 6 UF Module 2

2.2 GEMMA Unit.

The GEMMA Unit is comprised of a sample input assembly, an electrospray module, a differential mobility analyzer, a condensate particle counter and the controlling computer and associated software. The integral parts are described in the paragraphs following in this section with a flow schematic included in Figure 7.

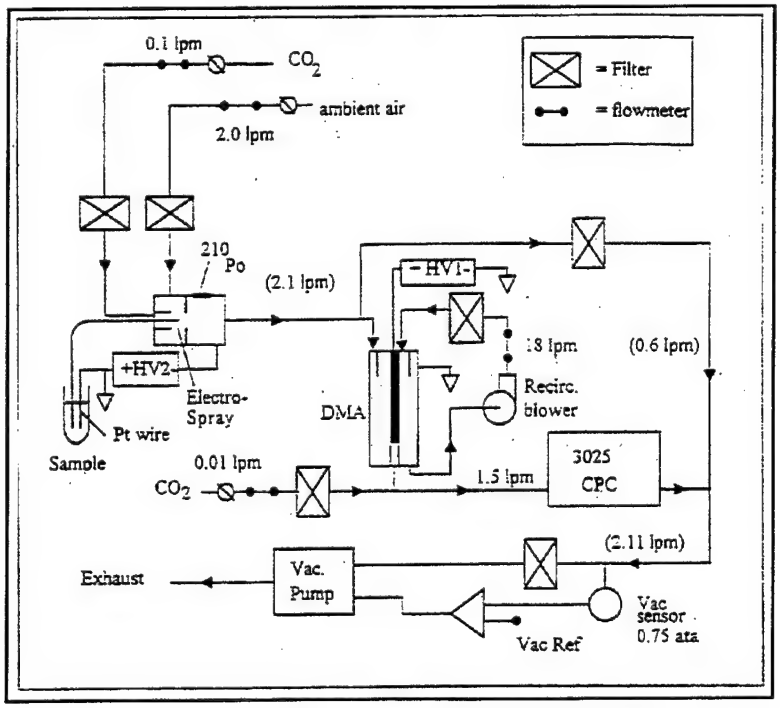

Figure 7 Flow Schematic of GEMMA Detector

2.2.1 Sample Input.

The inlet tube for the GEMMA is a fused-silica capillary of 25 μm inside diameter (I.D.) and 150 μm outside diameter (O.D.). The capillary is inserted into a section of PEEK tubing, of appropriate I.D., and the PEEK tubing is swaged into the front orifice of the electrospray unit. The sample is aspirated into the electrospray module by vacuum generated by a vacuum pump. The vacuum pump provides the flow parameters for sample passage through the GEMMA.

2.2.2 Electrospray.

The electrospray module subjects a conductive liquid to a strong electric field. The field produces a cone that emits a fine jet that then breaks up into small droplets and forms a fine plume. In the electrospray unit of the GEMMA, the droplets electrical charge is reduced to a single charge or is neutralized by using an alpha particle emitter that ionizes the surrounding air. To eliminate the possibility of the breakdown (corona discharge) of the air in the plume, as caused by the high electric field, the spray tip is surrounded by a flow of CO_2, which will prevent corona discharge.

2.2.3 Differential Mobility Analyzer (DMA).

The DMA separates particles by their electrical mobility in air. The sample stream flows through a gap between a rod and a cylinder with an electrical potential between the two. Particle mobility, which is related to size and charge, will either pass particles through the DMA or impinge on the walls. With singly charged particles, which are generated by the electrospray, the mobility becomes a direct measure of the particle size.

2.2.4 Condensation Particle Counter (CPC).

In the CPC, the sample particles flow in tandem with a saturated working fluid of butanol. The nanosized particles initiate the condensation of the butanol and the stream is then cooled. A standard optical counter can then count the butanol-condensed particles and the results are displayed via the supplied software.

2.3 Computer Software and Hardware.

The GEMMA is currently controlled with a 486 PC running the Scanning Mobility Particle Sizer (SMPS) software developed by TSI, Inc., St. Paul, MN. There are a number of options for displaying and interpreting the data with instrument software. The particle data is classified by one of the following values:

- Number – number of particles
- Surface – surface area of particles (assuming round spheres)
- Volume – volume of the particles (assuming round spheres)

The units displayed by the SMPS can be setup to display the following measurements:

- Counts – raw data without any corrections
- Conc. dW/dlgDp – normalized concentration, useful when comparing information to other instruments
- Conc. dW – the concentration within a particle size bin
- Log dW/dlgDp – vertical scale compressed to $\log_{10}$
- Conc. (W%) – the concentration as a percentage of total concentration
- Cumulative W – particle concentration in a cumulative format
- Cumulative W% – particle concentration in a cumulative percentage format

The data is displayed by choosing the options available and presenting the data as graphs or tables on the controlling PC or exporting the data into another spreadsheet program for alternate presentation capabilities.

3. Summary of Conclusions and Recommendations

There were a number of improvements to the IVDS that enhanced the performance of the system. The electrospray upgrade to the GEMMA unit increased the sensitivity of the detector approximately 10 fold (see Section 4.1). A pressurizable chamber to help eliminate capillary plugging was purchased (see Section 4.1).

A new CPC was identified that would improve the detection limits of the GEMMA unit by 30 fold. When funds are available, this improvement to the CPC should be purchased and incorporated.

When the ultrafiltration modules become more automated, a pump and autosampler have been identified for incorporation (see Section 4.3). In addition, there

are high molecular weight filters that may be available for the second module to the UF that will minimize absorptive losses of viruses in the filter (see Section 4.3).

The MS2 bacteriophage was found to be an excellent virus standard. The virus is stable in solution and is not affected by dilutions. MS2 is well characterized in the literature and is of a size, 26 nm, which works well with ongoing GEMMA analysis procedures. The other virus tested, an e-coli T7bacteriophage, was analyzed and is available as another standard (see Section 5).

The GEMMA unit was extremely reliable in its ability to count small quantities of viruses and continues to present a high signal/noise ratio around virus peaks. In addition, the GEMMA analysis of the particle size standards, obtained from Duke Scientific, presented results that were very close to the quoted size distribution (see Section 5).

The UF modules were tested by the successful removal of ~1% thyroglobulin from a solution while incorporating the appropriate buffer for GEMMA analysis (see Section 5).

4. Recent Improvements to the Integrated Virus Detection System

An improved electrospray module has been installed in the GEMMA for higher detection sensitivities. An upgraded CPC has been identified, but is not available at this time. The major improvements to the GEMMA are described in Section 4.1 and future upgrades are discussed in Section 4.2.

The UF modules 1 and 2 are currently on-line and are undergoing proveout studies involving the analysis of standards, known virus samples and known virus samples mixed with various proteins. Planned future upgrades to the UF module are discussed in Section 4.3.

4.1 Major Upgrades to the GEMMA Detector.

An improved electrospray apparatus was obtained from TSI and integrated into the front end of the detector. The new electrospray positions the inlet capillary closer to the aperture plate. In addition, the aperture plate is smaller on the new apparatus. These spatial improvements allow a greater percentage of the sample stream to be processed by the detection hardware. Several other improvements, internal to the electrospray, were also made that are remaining TSI, Inc. proprietary information at this time. These improvements have been shown experimentally to increase sensitivity 10-fold.

A pressurizable chamber, shown in Figure 8, which holds the GEMMA sample input container, or the third UF module, has been purchased from TSI. This chamber allows positive air pressure (a few psi) to be applied to the sample liquid. This is a

simple and effective way to eliminate bubbles that can otherwise vapor lock the GEMMA input capillary.

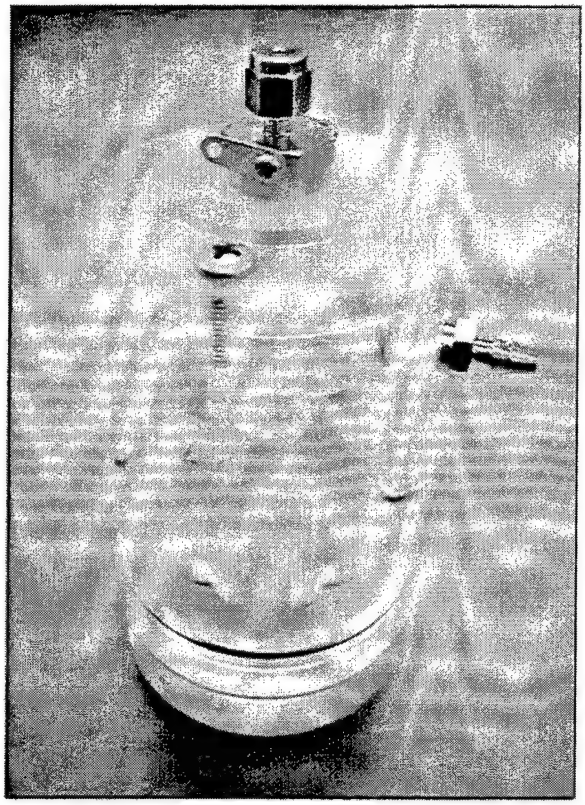

Figure 8 Pressure Chamber for Positive Sample Feed in GEMMA

A shortening of the detector capillary input has been implemented to greatly reduce the lag time between measurements and reduce complications with extremely large viruses.

4.2 Planned Upgrades to the GEMMA Unit.

A CPC component exchange (model 3010 to replace the current model 3025), that will increase sensitivity a further 30-fold, has been determined.

4.3 Planned Upgrades to the UF Stage.

CDA's have been signed, discussions have been held with, and a quotation has been obtained from SciLog, Inc., a supplier of a patented UF pump, that incorporates sophisticated regulating features, alarms, and controls for external switches and valves, which will provide superior performance and ease of automation in the next generation UF.

Negatively charged membranes, which will minimize loss of virus via adsorption, having the correct poresize (1 million Dalton MWCO) were discussed with Pall Filtron. These membranes will be acquired and tested in module 2 of the UF apparatus.

An autosampler, with an off-the-shelf cost of under $5,000, for queuing of density slices with automated input into the UF stage, was sourced.

4.4 Recommended Long Term Upgrades to the Integrated Virus Detection System.

The next stage in the development of IVDS are the experiments and processing of the zonal centrifugation stage, development of a cartridge for the UF module, and linking the systems together into an automated unit.

5. Laboratory Testing of the Integrated Virus Detection System

The laboratory work focused on testing the UF-GEMMA tandem's ability to purify and detect viruses at various concentrations and levels of background impurities. Several standard virus samples, of known pedigree, were analyzed. These viruses were used as markers in the purification and concentration studies that follow. A range of impurity levels were investigated in this study (up to 0.5% cesium chloride and 1.0% thyroglobulin) in order to ensure that the worst possible cases were addressed. Also tested were polystyrene sphere standards, of a known particle size, to determine the accuracy of the sizing of unknown samples.

5.1 General Test Protocols.

To process a sample through the GEMMA analyzer, the following steps should be considered as a guide. Since most samples processed to date contained varying degrees of contamination, orders of magnitude difference in virus concentration were artificially produced as samples for laboratory studies. A general decision tree for processing samples in the IVDS is shown in Figure 9.

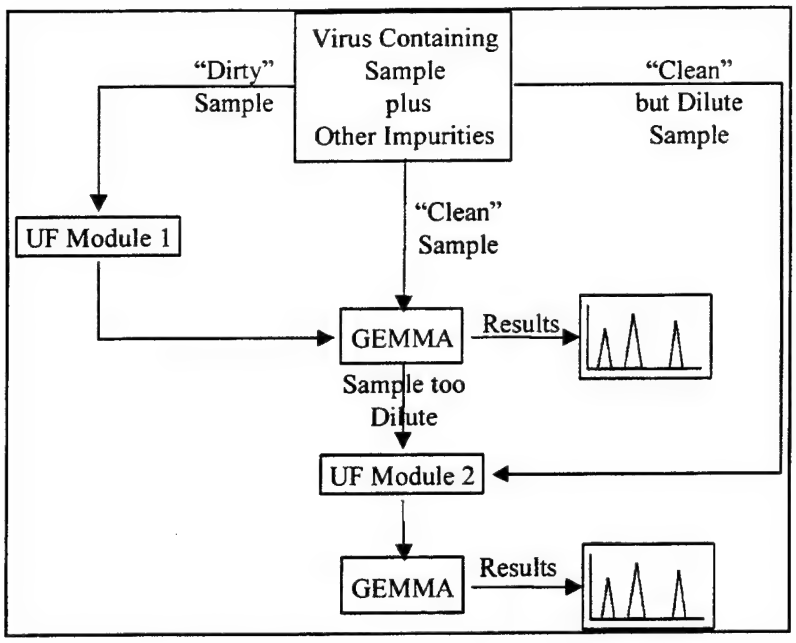

Figure 9 Decision Tree for IVDS Analysis

When a sample is received, it must be determined if the sample is "clean" enough to be analyzed by the GEMMA. The criteria for GEMMA analysis are the following:

1. Interferences from biological contamination must be low enough to allow GEMMA analysis. High protein concentrations will swamp the detector display and cover over any virus signals of interest. If the protein concentration is deemed too high, the sample needs to be processed through module 1 of the UF apparatus.

2. Any salt solution contamination, with a high concentration, will produce the same masking of the virus signal of interest as a high protein concentration. A high salt concentration will require processing through module 1 of the UF apparatus.

In addition, the amount of virus contained in the sample needs to be high enough to count when the sample is aspirated into the GEMMA detector. The easiest way to determine if there are enough viruses to count is to run a GEMMA analysis on the sample, as the analysis is easy to perform and can be completed in a few minutes.

The following protocols were used as a general guide for the analysis of virus containing samples in the following studies.

Protocol I:

1. Determine if the sample is clean enough for direct processing in GEMMA.
2. A clean sample can now be analyzed in the GEMMA detector. Results can be printed or archived.
3. If sample is too dilute, it will require the further concentration in module 2 of the UF apparatus. Module 2 can reduce the volume significantly to allow for the successful analysis and interpretation of the virus sample.

Protocol II:

1. If the sample is too dirty for direct processing in GEMMA, it will require pre-analysis processing.
2. The contaminated sample is processed in module 1 of the UF apparatus by adding enough buffer in the filter loop to replace any interfering species. Typically the samples to date have been processed in module 1 with a 40:1 (buffer to solution) volume addition. The 40:1 addition has removed the contaminating species and allowed for sufficient concentration of the virus sample to be processed in the GEMMA detector.
3. A clean sample can now be analyzed in the GEMMA detector. Results can be printed or archived.
4. If sample is too dilute, it will require the further concentration in module 2 of the UF apparatus. Module 2 can reduce the volume significantly to allow for the successful analysis and interpretation of the virus sample.

Protocol III:

1. If the sample is known to be clean but not very concentrated, it will require pre-analysis processing.
2. The clean dilute sample can be placed directly into module 2 of the UF apparatus and concentrated in one pass through the module. Typically the sample will be concentrated to the fullest extent possible with the 2nd module. If, however, the sample should become too concentrated, where it could plug the inlet capillary in the GEMMA, it is a simple matter to dilute back slightly with buffer solution.
3. The clean sample can now be analyzed in the GEMMA detector. Results can be printed or archived.

5.2 Processing and Analysis of MS2 from Cell Culture.

One key, non-trivial step in this work was to obtain a highly purified preparation of MS2, which could be used to insert a known amount of virus into the samples that contain standardized background components. MS2 preparations obtained from all previous sources contained high levels of background components, including proteins (even very high MW proteins), bacterial pili and fragments thereof, and salts, all of which

cause interfering signals in the high-sensitivity GEMMA detector. In the process of producing highly purified MS2, some very interesting results were seen from the partially completed IVDS system, which point to capabilities that surpassed prior estimates.

Some of the tests documented in this report (Sections 5.4, 5.5 and 5.6) discuss the analysis of MS2 bacteriophage that was purified using a purification and detection protocol that is more nearly the same as the protocol to be used in the final IVDS than any sample analyzed to date. Although the IVDS density-gradient centrifugation stage is not yet online, the sample of MS2 that was obtained for these tests was purified by density-gradient centrifugation prior to our obtaining it. Note that a further 300-fold increase in sensitivity will be effected in the next few months via integration of a new electrospray apparatus and a different model CPC unit, as outlined in Section 4.3.

Upon receipt of the purified sample, it was repeatedly dialyzed in an ammonium acetate buffer, since the ammonium acetate is virtually invisible to the GEMMA detector. Dialysis removed any remaining traces of non-volatile salts (phosphate, cesium chloride, etc.), together with any other soluble impurities, such as proteins, that give rise to a "salt peak" at low sizes, 10 nm or less. Any features above this are due to actual particles, including viruses, aggregates, viral fragments, and any non-viral impurities in the 10-180 nm size range.

5.3 Standards and Specifications.

The reference particle size standards used in this study were Certified Nanosphere Size Standards purchased from Duke Scientific Corp. The standards are monodispersed polystyrene microspheres that were calibrated with methodology traceable to the National Institute of Standards and Technology (NIST). The standards certified mean diameters were 73 nm and 54 nm respectively.

5.4 Cultures Tested.

Cultures tested from known sources were an antigen grade, MS2 bacteriophage, obtained by Envirion and a E-Coli, T7 bacteriophage purchased from ATCC. The antigen grade MS2 arrived at a concentration of $1x10^{13}$ pfu/ml and was density gradient centrifuged and dialyzed. The T7 bacteriophage arrived in a freeze-dried state, at a concentration of $2x10^{8}$ pfu/ml. The freeze drying also retained the growth media, which was mainly composed of skim milk products.

5.4.1 Background on MS2 Bacteriophage.

In other MS2 samples that have been previously analyzed, evidence of high dimerization tendencies has been noted. It is well known that electron microscopy gives an MS2 particle diameter of 24-27 nm. However, what is perhaps less generally known, or recognized as important, is the arrangement of MS2 virions in pairs on the bacterial pili, like barbells, as shown in the TEM micrograph of Figure 10.

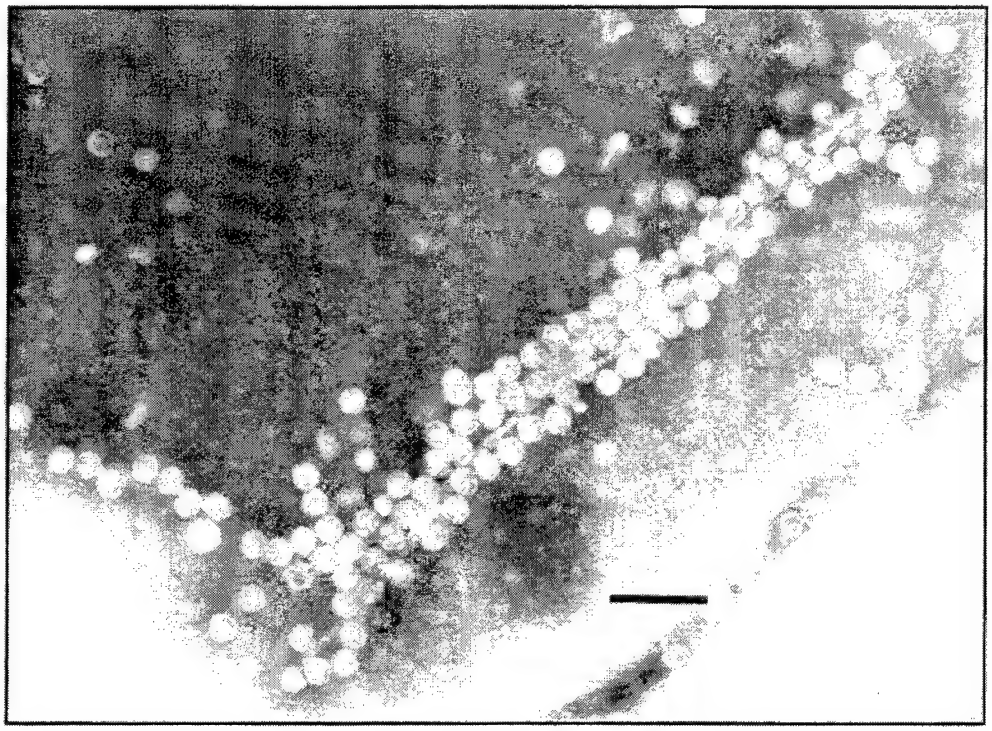

Figure 10 TEM Micrograph of MS2 Bacteriophage (bar represents 100 nm)
(Dr. Hans Ackermann, Universite Laval, Canada)

Such micrographs suggest why we might see a size corresponding to an MS2 dimer, in the GEMMA detector data. If the viruses are still complexed to the pili at the time the structure reaches the electrospray, it might happen that the electrospray action breaks the pilus along its length, freeing viruses in pairs. In previous work, very long molecules, particularly those containing weak linkages, have been shown to break up along their length in the GEMMA electrospray, yielding sizes corresponding to the fragments.

In previous work on MS2, the dimer peak (as we interpret it) always appears at a size between 37 and 41 nm. The dimer does not appear as a particle size of 48 nm to 52 nm (the size of two monomers attached together) because the particle size algorithm in the CPC fits measured sizes into an apparent spherical diameter. This fitting algorithm yields the 37 nm to 41 nm in the graphs. In at least one virus we have seen evidence of four peaks corresponding to monomer, dimer, trimer and tetramer, with the peak positions (diameter D) fitting well to a formula:

$$D = D0\ N^{a},$$

where the exponent a is 0.39. One should expect the exponent a to fall between 1/2 and 1/3, corresponding to cylindrical- and spherical-like growth with aggregation number N, as observed.

5.4.2 Background on T7 Bacteriophage.

The T7 bacteriophage is described as a P-22-like phage. The head is approximately 60 nm in diameter and is icosahedral in symmetry. The tail, which has six short fibers attached, is approximately 17 nm long and 6 nm wide. Figure 11 shows a photomicrograph of the bacteriophage.

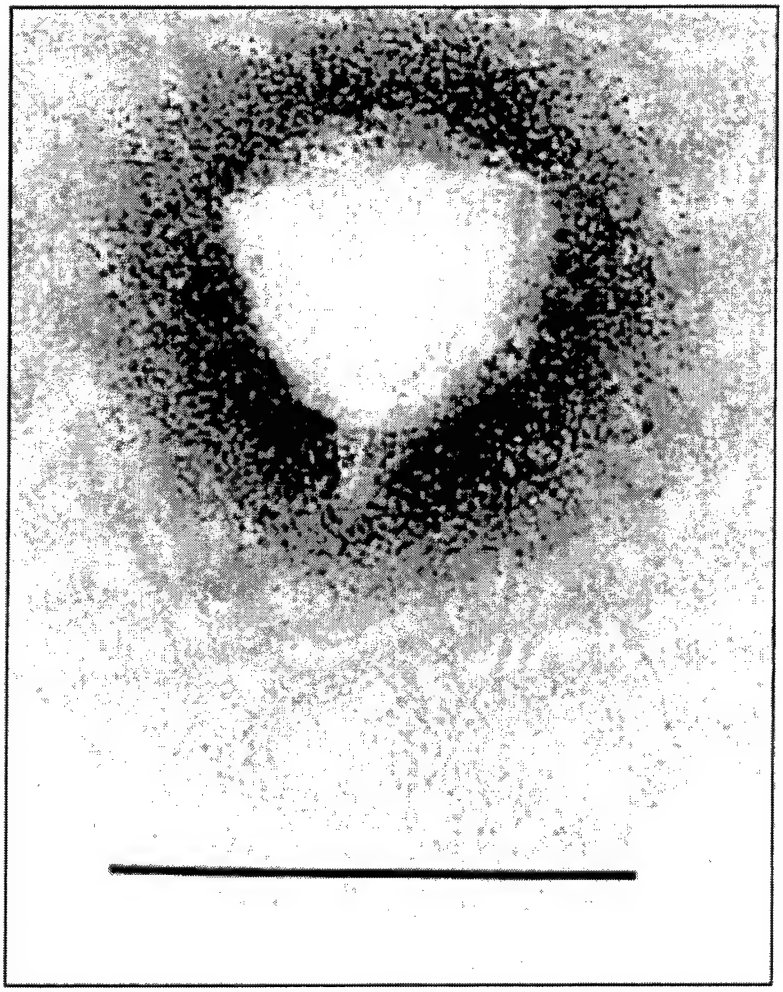

Figure 11 TEM Micrograph of T7 like Bacteriophage (bar represents 100 nm)
(Dr. Hans Ackermann, Universite Laval, Canada)

5.5 Test Matrix.

Table 1 presents an overview of the tests performed using the ultrafiltration modules and the GEMMA analyzer.

Table 1 Test Matrix

Section	Species	Objective	Comments
5.4.1	MS2 bacteriophage	Analysis of virus standard Analysis of signal/noise ratio	Protocol I Individual viruses – 26 nm Concentration: 10^{13} to 10^{8} pfu/ml
5.4.2	T7 bacteriophage	Characterization of alternate virus species	Protocol I Head – 60 nm Tail – 17 nm Concentration $2x10^{8}$ pfu/ml
5.4.3	Polystyrene spheres	Analysis of particle size standard	Protocol I Standards – 54 nm and 73 nm Number concentration ~10^{12} particles /ml
5.4.4	Cesium Chloride	Analysis of standardized salt solutions	Protocol I Salt peak position vs. impurity Concentrations of 0.5% and 0.05% CsCl
5.4.5	MS2 bacteriophage	Ultrafiltration modules proveout	Protocol III Purification and concentration of virus containing solutions Concentration: 10^{13} to 10^{8} pfu/ml
5.4.6	Thyroglobulin with MS2	Ultrafiltration modules proveout	Protocol II Purification and concentration of virus containing solutions Removal of protein contamination 1% thyroglobulin plus $5x10^{10}$ pfu/ml MS2

5.6 Test Results.

5.6.1 Virus Standard – MS2 Bacteriophage.

The MS2 bacteriophage was chosen as a standard for its stability and uniform size. Although the virus was received as a dimer, due to the culture being fresh, the dimers quickly separated in monomers and stabilized in solution. This section shows the results of tests with MS2 virus concentrations between $1x10^{13}$ pfu/ml and $1x10^{8}$ pfu/ml.

5.6.1.1 Results of Undiluted and 10-Fold Diluted Samples of MS2

Figure 12 is the scan of the sample at full strength, which, after the dialysis protocol, was at a concentration of $1x10^{13}$ pfu/ml (a very high concentration which attests to the effectiveness of the purification/concentration protocol). The "salt peak" is centered on 10 nm. A small peak is visible at 26 nm, with a dominant peak occurring at 37 nm. A closer view of the MS2 signal is presented in Figure 13, which spans the 19-45 nm region.

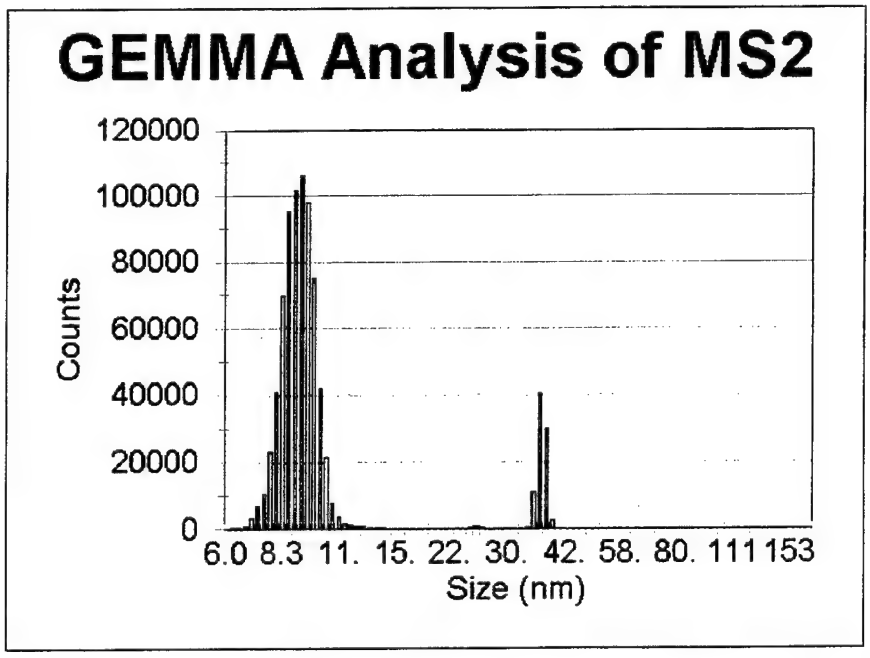

Figure 12 Antigen Grade MS2 (presented as a dimer) at a Concentration of $1x10^{13}$ pfu/ml

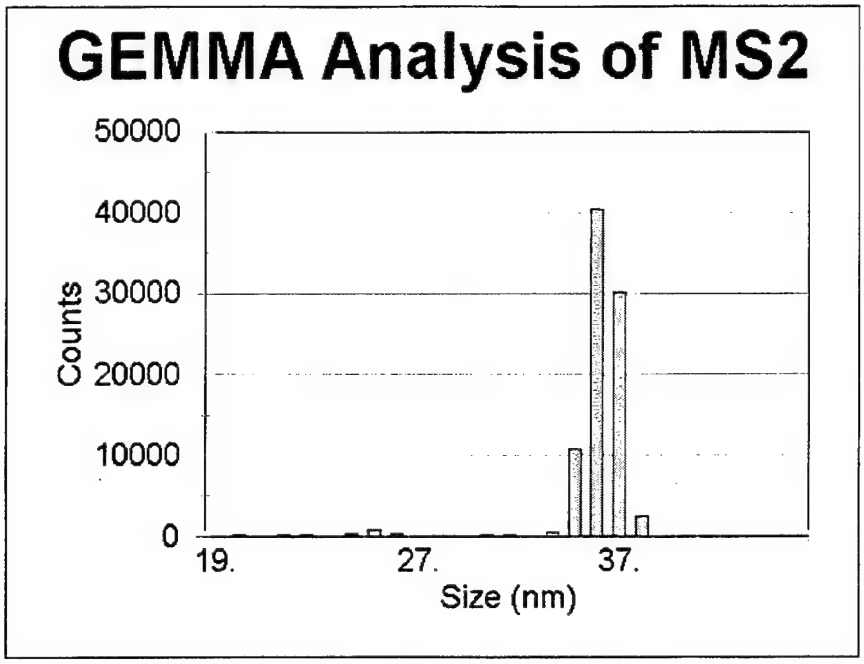

Figure 13 Close-up View of Figure 12 from 19 to 45 nm

The sample was then diluted ten-fold and analyzed again. The effect of dilution is different for the "salt peak" as compared to real particles. A 10-fold dilution shifts the position of the salt peak by a reduction of $10^{1/3} = 2.15$ in size, in this case from 10 nm to about 5, thus increasing the signal/noise ratio in the region 10-20 nm. For a real particle such as a virus or viral fragment, the position remains approximately fixed but the intensity is reduced, approximately 10-fold. The resultant scan is presented in Figure 14.

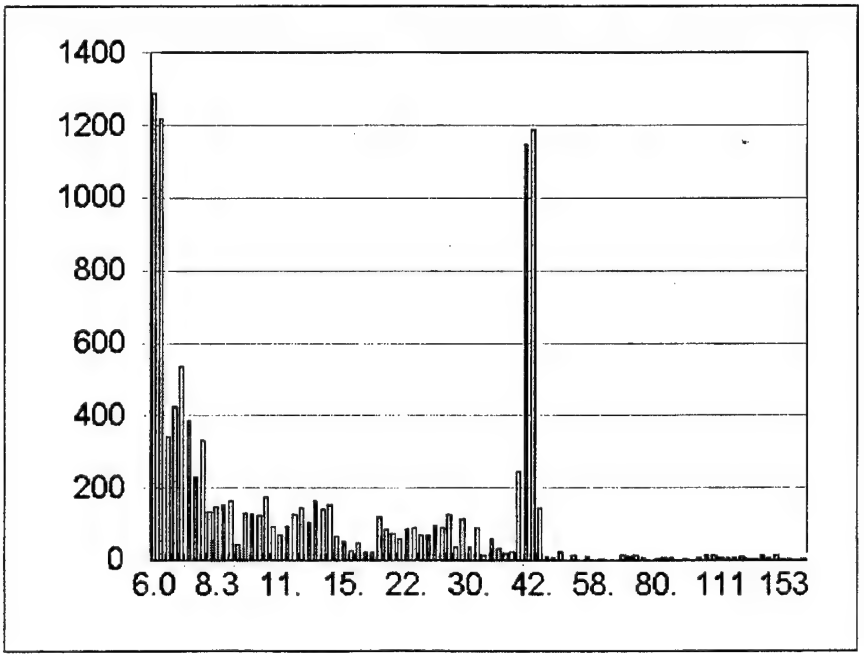

Figure 14 MS2 Antigen Grade MS2 (Figure 12) Diluted 10-Fold to $1x10^{12}$ pfu/ml

5.6.1.2 Analysis of Data from Undiluted and 10-Fold Diluted Samples of MS2

Because of the increase in signal/noise ratio in the 10-20 nm range, Figure 14 reveals some small features that are not present in Figures 12 and 13 on the undiluted sample. There is some noise in this range, but previous experience with MS2, and with RNA, has shown the areas to look in for information on viral fragments. In particular, as exemplified in the Figure 15 for a two-year-old preparation of MS2, we generally see a peak at 13 nm, which we believe corresponds to a proteinaceous subunit of MS2. The MS2 coat protein, which has the PBD identification code 1msc and comprises 69% of the viral mass, is known to dimerize, forming dimers which from X-ray analysis are known to have a length of 12.9 nm [4,5]. These dimers then assemble into units containing 5 dimers, which are referred to as pentamers, also of length 12.9 nm. The viral coat is composed of 12 such pentamers plus an additional 12 dimers, to make the icosohedral structure. Clearly, based on a 26 nm diameter (viz., 13 nm effective radius) of the entire particle, and the fact that the coat makes up 69% of the mass, all three dimensions of the pentamer must be approximately 13 nm, and should therefore be registered in the GEMMA at around 12-13 nm. Since the virus assembles by assembly of the pentamers, it is reasonable to assume that it may well disassemble into these same units, to a large extent. In view of the fact that aged virus preparations have thus far shown increased peak intensities at 12-13 nm, with qualitative correlation between age and intensity, we believe that the intensity at 12-13 nm is probably a sign of fragmentation. Its absence in fresh MS2 preparations indicates that it is not created by breakup in the electrospray, and this is supported by experiments on several protein complexes, which also remain intact.

[4] L. Holm and C. Sander (1996) Nucl. Acids Res. 24, 206-210.
[5] L. Holm and C. Sander (1994) The FSSP database of structurally aligned protein fold families. Nucl. Acids Res. 22, 3600-3609.

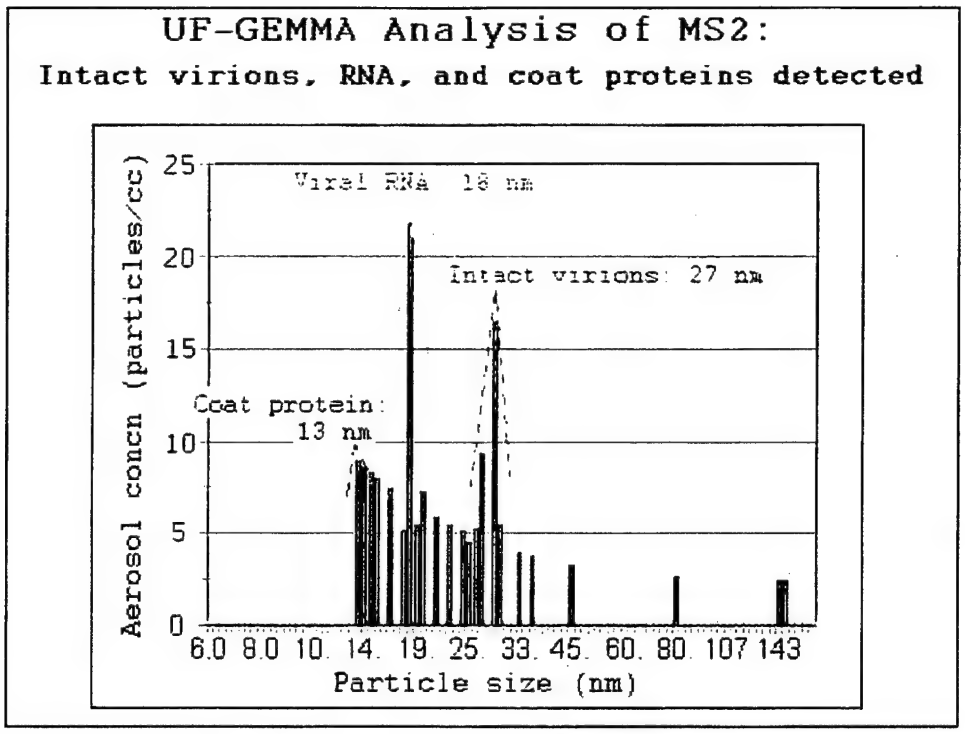

Figure 15 MS2 Preparation – Two Years Old

The GEMMA size measured for the RNA of MS2 can be estimated accurately from published reports of ssDNA analysis using the GEMMA. The formula derived from the publication of Mouradian et al[6]. for ssDNA or RNA is:

$$D = 1.11\, X^{1/3},$$

where X is the number of nucleotides. The MS2 genome is 3,569 nt in length, so that the formula yields D=17 nm. The formula was derived for rather short ssDNA strands, and we have found in previous work that aged MS2 shows a peak closer to 18 nm, which we attribute to viral RNA.

Thus within the limitations set by the noise in the above data, we can deduce evidence from Figure 15 for a small protein subunit peak centered at 13 nm, and an equally small RNA plot centered at 18 nm, in addition to the monomer and dimer virus peaks at 26 and 37 nm. The fragments, protein subunit and RNA, are clearly an order of magnitude lower in intensity (and thus concentration) than the monomer+dimer intensity, indicating a low degree of fragmentation under the conditions applied. It should be noted that even in the diluted sample, any data below 10 nm is meaningless, due to the entrance of soluble proteins below 10 nm.

[6] Mouradian et al, Anal. Chem. 69 (5): 919.

5.6.1.3 Analysis of Signal/Noise Ratio for Undiluted Solutions of MS2

For a quantitative analysis of the signal/noise ratio in Figure 13, we must examine the raw data because this is far more accurate than visual inspection of the graphs. Table 2 shows the actual detector data.

Table 2 Raw Data for Signal/Noise Ratio Analysis of Antigen Grade MS2

Channel Particle Size Midpoint(nm)	GEMMA Counts
23.7	87
24.6	364
25.4	877
26.4	269
27.4	49
28.4	66
29.4	54
30.5	116
31.6	107
32.8	75
34.0	533
35.2	10800
36.5	40359
37.8	30052
39.2	2571
40.7	77
42.1	0
43.7	33
45.3	0
47.0	0
48.7	0
50.5	15
52.3	0
54.2	0
56.2	14
58.3	0
60.4	0

As shown in Table 2, there are about 40,000 counts at the main peak channel, 36.5 nm (corresponding to virus dimers), and a total of 80,000 counts over the 3-channel spread of the peak. What was not accurately shown in the graphical analysis but is evident from the tabulated data is the extremely low background just outside of the peak region. Indeed, in the larger particle size direction, only 3 channels beyond the peak, the intensity drops to literally zero counts per channel, and 4 of 5 consecutive channels have zero counts per channel. This indicates two things:

1. The resolution of the peak is very sharp
2. The signal/noise ratio is extremely high, much higher than the 2,000:1 previously estimated.

5.6.1.4 Results of Further Dilute Solutions of MS2

A concentration of $2x10^{10}$ pfu/ml was sampled straight into GEMMA, yielding fairly high counts (several hundred per channel at the peak position), and a signal/noise ratio of about 25:1, as shown in Figure 16. Note that the virus concentration, as well as the GEMMA amplitude, are about 2 to 2-1/2 orders of magnitude lower than in the $1x10^{13}$ pfu/ml sample analyzed in Section 5.6.1.1 (which showed about 40,000 counts/channel at the peak).

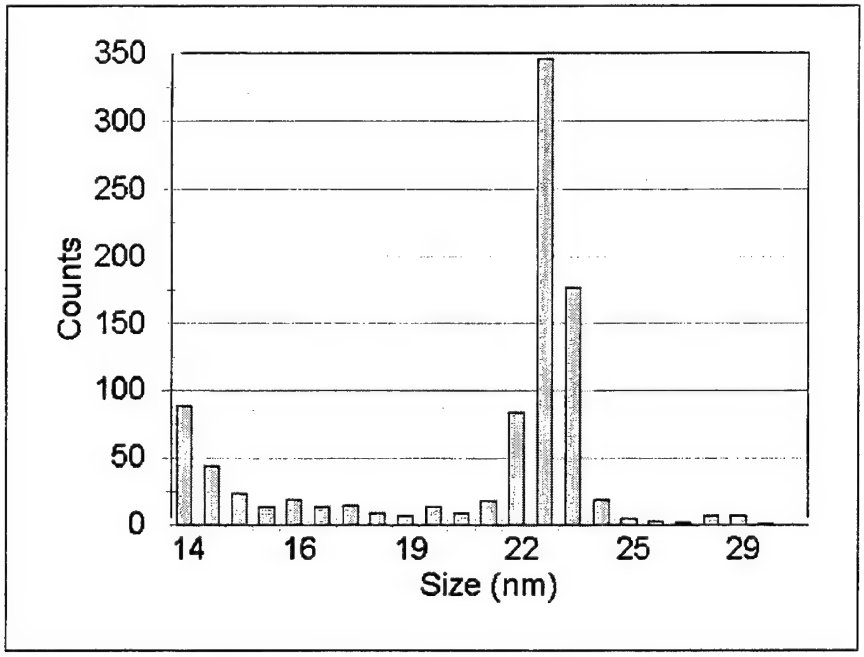

Figure 16 GEMMA Scan of $2x10^{10}$ pfu/ml MS2

After diluting an additional 100-fold, a $2x10^{8}$ pfu/ml solution was sampled directly into the GEMMA. This low concentration of virus was detected with a small number of counts, but still maintained a exceedingly good signal/noise ratio, as shown in Figure 17. Note how the salt peak due to remaining soluble impurities has been pushed off the chart axis, which extends below 3 nm.

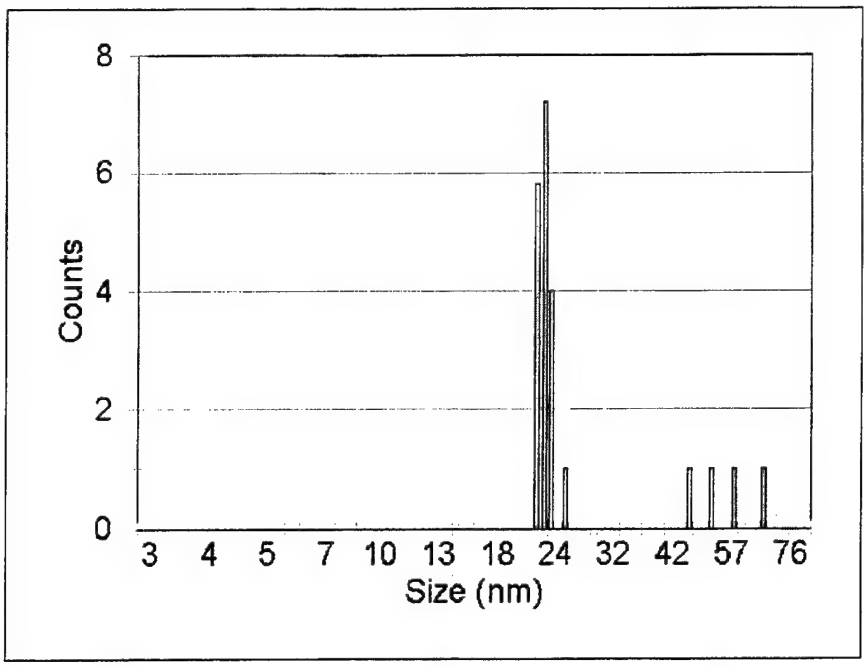

Figure 17 GEMMA Scan of $2x10^8$ pfu/ml MS2

5.6.1.5 Analysis of Further Dilute Solutions of MS2

Figure 17 demonstrates the feasibility of detecting viruses at low concentrations and very small number counts, with levels of purification that yield zero counts in nearly all adjacent channels.

Thus, in summary, we have started with MS2 viruses buried in a bacterial culture and, by a combination of density-gradient centrifugation, simple ultrafiltration, and GEMMA detector analysis, detected the MS2 virus with a 15,000:1 signal/noise ratio. Such high signal/noise ratios are rare in experimental science, with any experimental probe, particularly when the starting point is a virus lying within a sea of biological background material.

5.6.2 Characterization of T7 Bacteriophage.

5.6.2.1 Results

In addition to the MS2 phage used in this study, we also obtained a sample of the T7 phage, from ATCC. This was reconstituted as a $2x10^8$ pfu/ml solution, according to the instructions provided with the material.

This was then diluted ten-fold, to $2x10^7$ pfu/ml, and run into the GEMMA. The T7 phage was clearly detected at 3000 counts/channel, as shown in Figure 18.

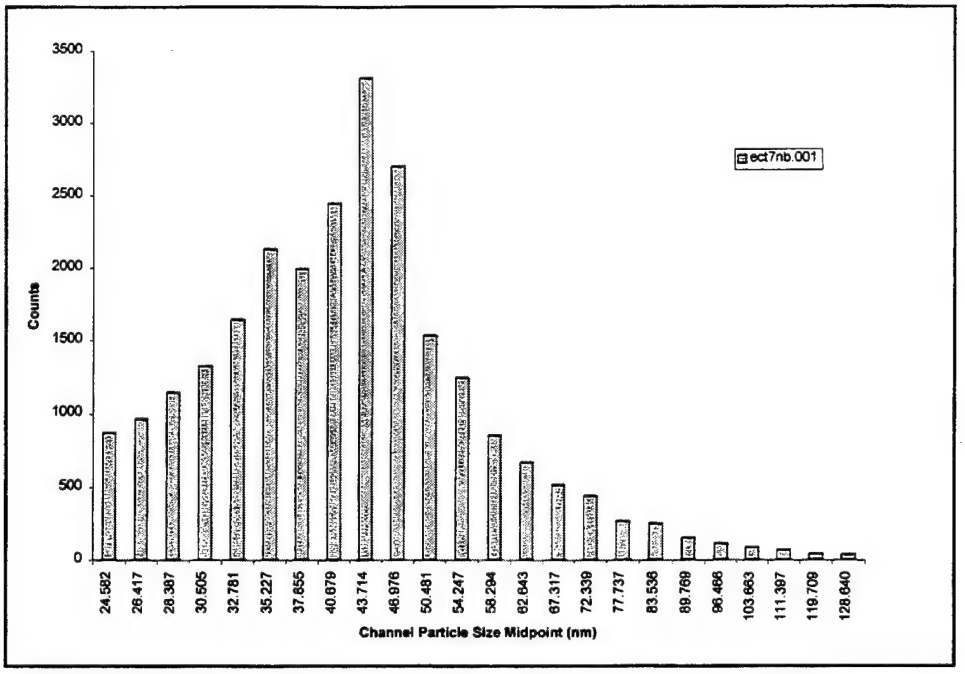

Figure 18 GEMMA Scan of T7 Bacteriophage

5.6.2.2 Analysis of T7 Bacteriophage

It is very interesting to note that according to the formula derived above for converting counts to particle concentrations, we estimate that the number concentration of virus particles is about 10^{10} particles/ml, whereas according to the information supplied by ATCC, the concentration should have been $2x10^7$ pfu/ml. This means that a single pfu corresponds to about 1,000 virus particles. Whether or not this figure has been inflated due to overly-conservative estimates of the virus potency by ATCC should be determined in the future, as the interesting issue of pfu-virion conversion comes to the foreground.

5.6.3 Particle Size Standards.

5.6.3.1 Results

Nanoparticle standards of average diameter 73 nm (±2.6 nm) and 54 nm (±2.7 nm) were obtained from Duke Scientific Corporation and analyzed by our GEMMA. At a number concentration of order 10^{12} particles/ml, corresponding to 0.05% by volume, these were easily detected, and sized in excellent agreement with the quoted size distribution. Approximately 1,400 and 4,000 counts were obtained in the peak region for the 73 nm and 54 nm standards respectively as shown in Figures 19 and 20.

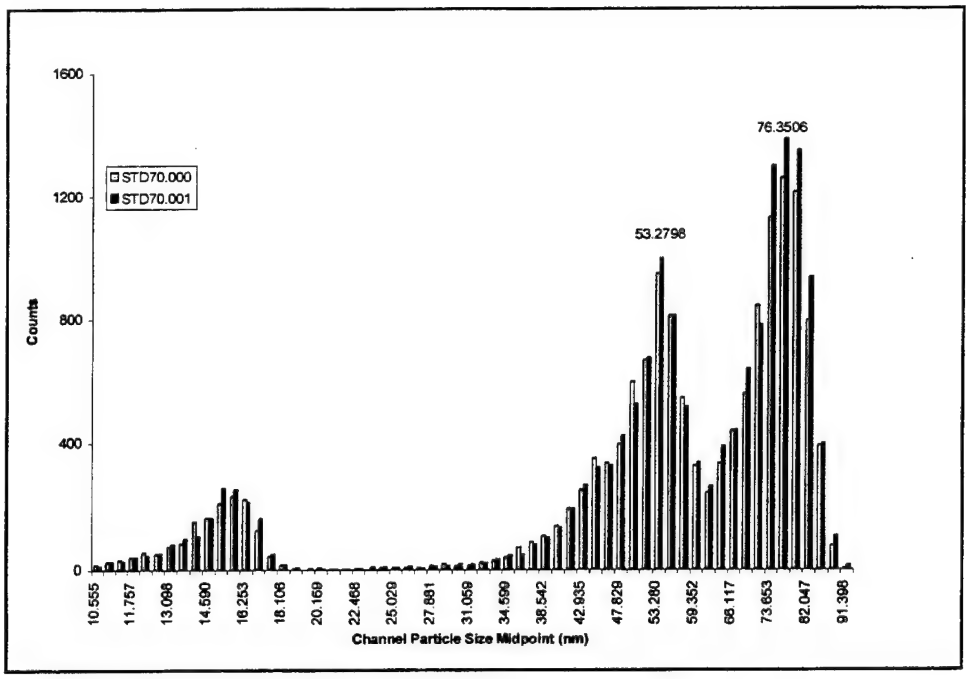

Figure 19 GEMMA Scan of 73 nm Polystyrene Standard Spheres

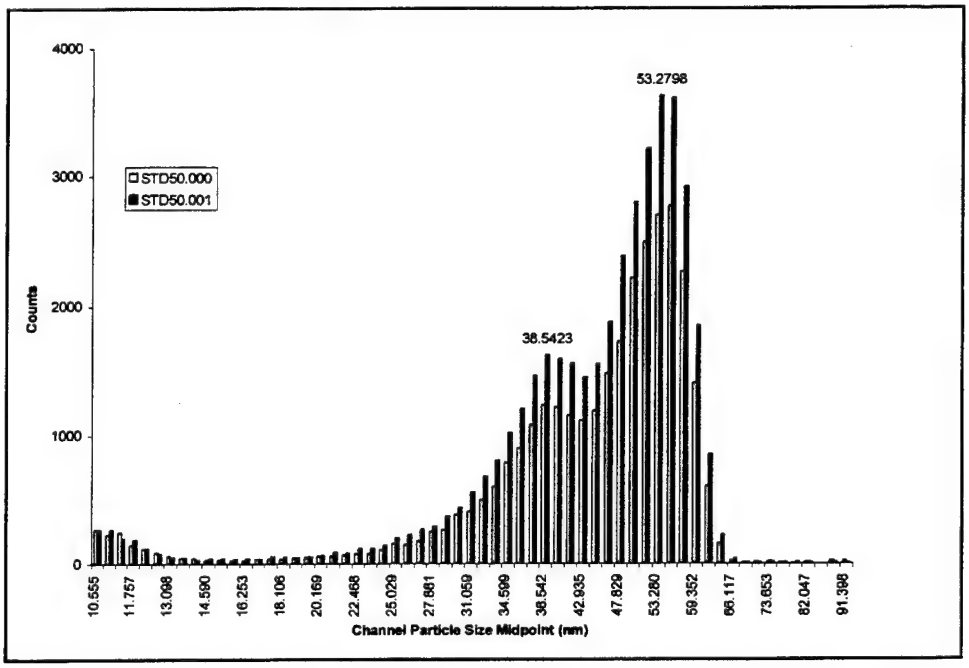

Figure 20 GEMMA Scan of 54 nm Polystyrene Standard Spheres

5.6.3.2 Analysis of Particle Size Standards

This analysis of standardized particles allowed us to develop a formula for converting from peak counts to particle number density. Approximately, the concentration in particles/ml = $2x10^8$ x (total number of counts in peak), under current operating conditions and normalized to a 60-second runtime. This sets a GEMMA lower detection limit, under current conditions, of about 10^9 particles/ml in UF-concentrated output. If the output volume of the 2nd module of the UF is 10 microliters, then the lower limit of detectable particle number count (as opposed to number concentration) is about 10^7 particles. In the worst case, 1 pfu equals 1 virus particle, but, in general, 1 pfu will comprise many virus particles. Indeed, for the case of the T7 virus analyzed in Section 5.4.2, it is likely that one pfu equals several thousand virus particles. Therefore the detection limit of 10^7 virus particles corresponds to something between 10^3 and 10^7 pfu's, with 10^5 pfu's probably being a typical case. With the new CPC operating schemes as discussed in Section 4.2, these detection limits could readily be reduced to below 10^3 pfu's, thus rivaling or exceeding that of the highly-capable PCR methods. Note that PCR methods handle only specific viruses for which sequencing information and primer molecules are available.

5.6.4 Standardized Salt Solutions.

5.6.4.1 Results

Cesium chloride solutions, with concentrations starting at 0.5% and proceeding downward in decades, were analyzed directly by the GEMMA without purification. The resultant scans are illustrated in Figures 21 and 22. The position of the resulting "salt peak" showed a cube root dependence on concentration, up to 0.05%. The relocation of the salt peak with a change in concentration is discussed in Section 5.6.4.2. For the sample at 0.5%, an effect of the salt concentration on conductivity of the solution, and thus operation of the electrospray, was easily seen. This was evidenced both as a departure from the cube root position and a perturbation in the shape of the peak.

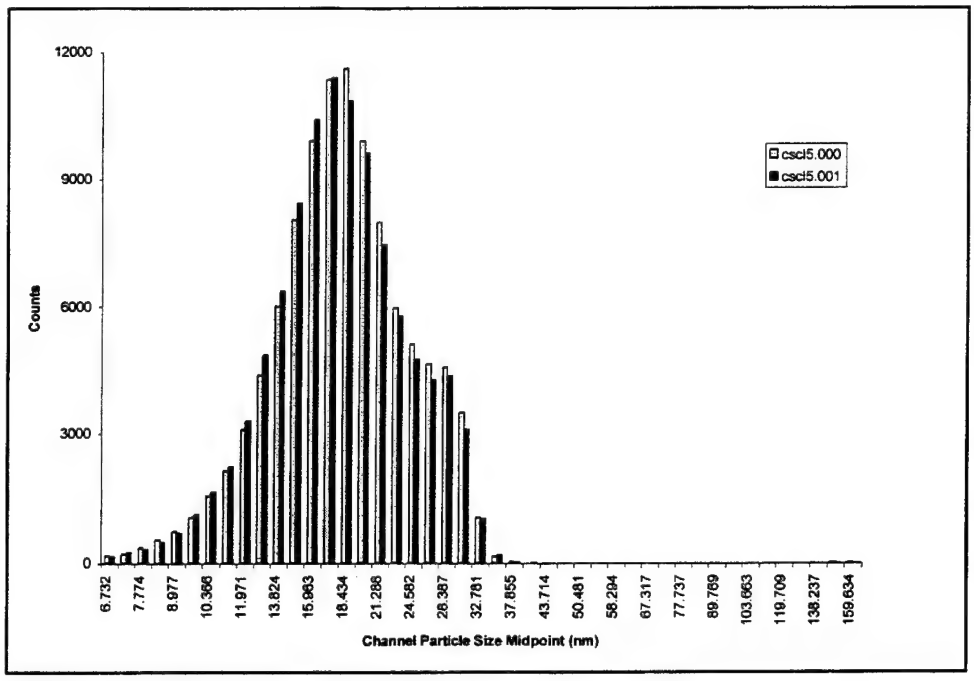

Figure 21 GEMMA Scan of 0.5% CsCl in Ammonium Acetate Buffer

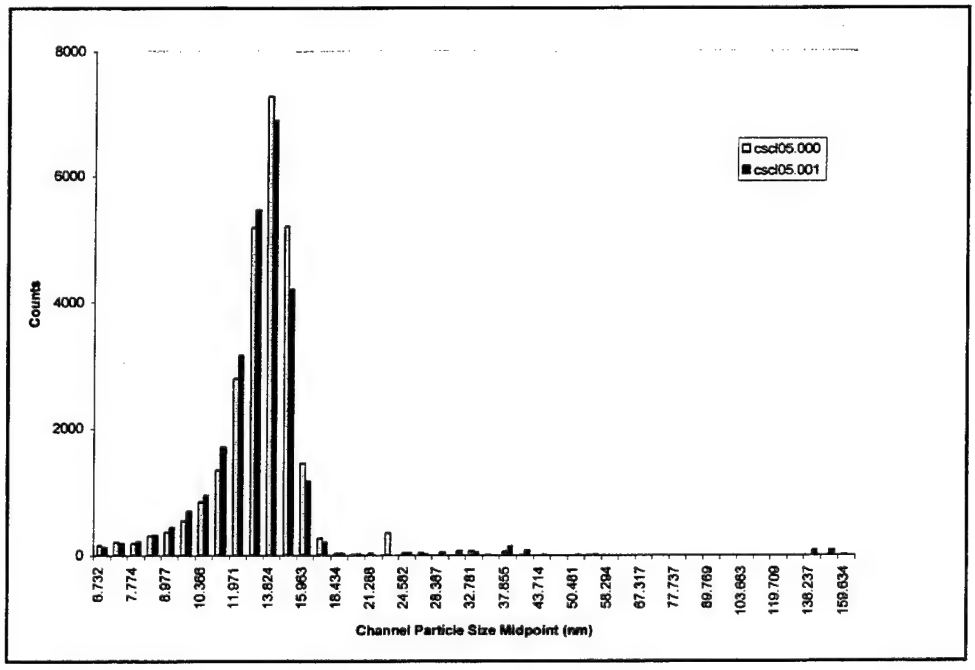

Figure 22 GEMMA Scan of 0.05% CsCl in Ammonium Acetate Buffer

5.6.4.2 Analysis of Standardized Salt Solutions

Assuming that the electrospray is still operating normally at 0.05% CsCl, we can calculate the droplet size in the electrospray from the peak position, 13 nm. Taking into account the density of solid (dried, in-flight here) cesium chloride, which is 3.99, the droplet size is calculated to be 265 nm, which fits very well with the expected droplet size on the order of 300 nm according to our operating conditions. This figure, 265 nm, will be used in subsequent computations herein for converting between salt peak position and impurity concentration.

5.6.5 Ultrafiltration-GEMMA Tandem Analysis with MS2 Test Results and Discussion.

Beginning with the highest starting concentrations of virus, and working down to the lowest investigated, we present results on the analysis of density gradient, centrifuge-purified MS2 with the newly constructed UF device, module 2. As this sample of MS2 was highly purified, the samples could be processed directly into module 2. Figures 23 and 24 show, respectively, the GEMMA response before and after application of the 2nd module, utilizing a 1M Dalton filter, where the starting concentration of MS2 was $2x10^{13}$ pfu/ml.

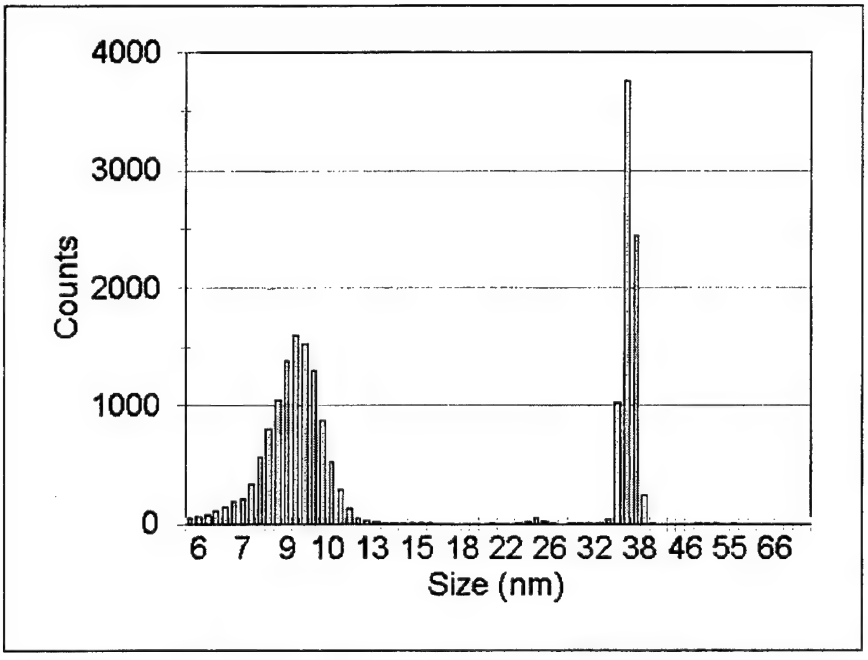

Figure 23 GEMMA Scan of $2x10^{13}$ pfu/ml MS2

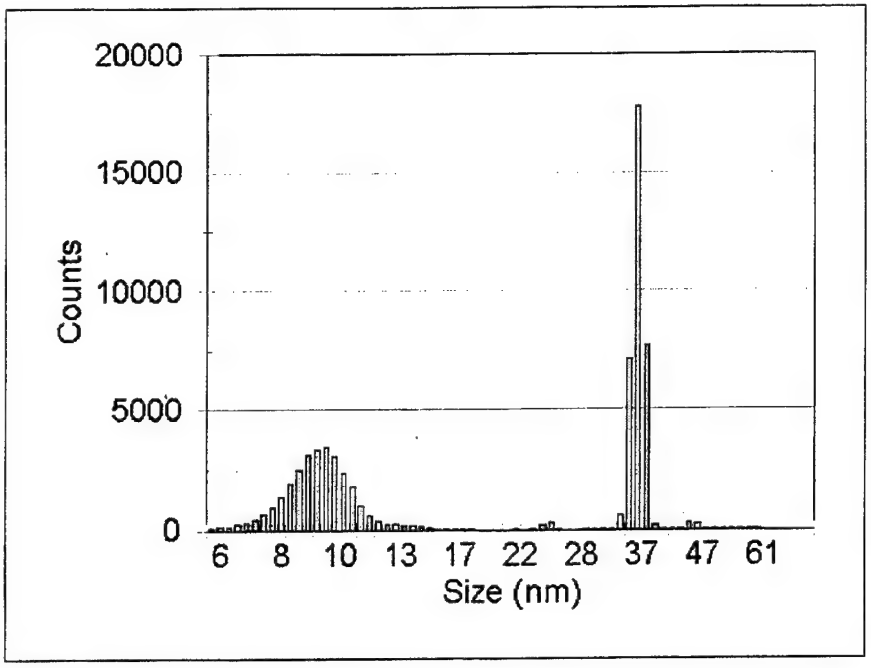

Figure 24 GEMMA Scan of $2x10^{13}$ pfu/ml MS2 after Application of 2nd Module

After one compression in the 2nd module, the ratio of peak amplitude to the amplitude at the highest "noise channel" (at 9.5 nm) increases from about 2:1 to about 6:1, with about 18,000 counts in the highest peak channel. Note that, since there was no diafiltration in this case, the number of counts in the "salt peak" (centered at 9 nm, approximately 2,000 counts in the highest channel) did not change. This serves to remind the reader of the importance of successive diafiltrations. That is, when one is simply pushing solution through the UF membrane, although the quantity of retentate liquid and thus the amount of non-viral impurities are being reduced many-fold, the actual concentration of these impurities does not change. These impurities can only be reduced by diafiltration, when clean buffer is added to dilute the impurities prior to a filtration step. In any case, the virus concentration is increased by a simple filtration without diafiltration, as seen in Figures 19 and 20, examined at the 37 nm position.

The increase in the peak amplitude at 37 nm after the compression is about 5-fold, from 3,600 to 18,000 counts per channel. This correlates well with the approximate 5-fold reduction in volume that was applied in the 2nd module step, namely reduction of the volume from about 200 to 30-40 microliters. Whenever a 5-fold reduction in volume results in a 5-fold increase in signal (in turn indicating a 5-fold increase in concentration), the virus retention by the UF module is very high, and nearly all the virus is being retained by the membrane and is present in the extracted liquid.

Starting with an MS2 concentration of $2x10^{11}$ pfu/ml and applying one compression in the 2nd module yields a superb signal/noise ratio, with amplitude dropping from approximately 1,000 counts/channel to literally zero counts/channel only 5 channels away from the peak center. This occurs on both sides of the peak, as shown in Figures 20

and 21. The difference between the two plots shown is that the 2nd module compression was carried out, in the Figure 22 plot, to about half the retention volume of that for the Figure 21 plot, yielding roughly 20 instead of 40 microliters. The doubling of the signal between the two conditions indicates that the GEMMA peak amplitude is roughly linear with final retention volume. This linearity again supports the conclusion that there is a high degree of retention of viruses.

The reader will note that the peak at 23 nm (MS2 monomers) has assumed dominance over the dimer peak at 37 nm between the initial sampling (month of August) of the 10^{13} pfu/ml MS2 bacteriophage, Figures 23 and 24, and the later sampling (Figures 25 and 26) of the virus with lower concentrations. Over the month's time, the dimers broke down into monomeric virions.

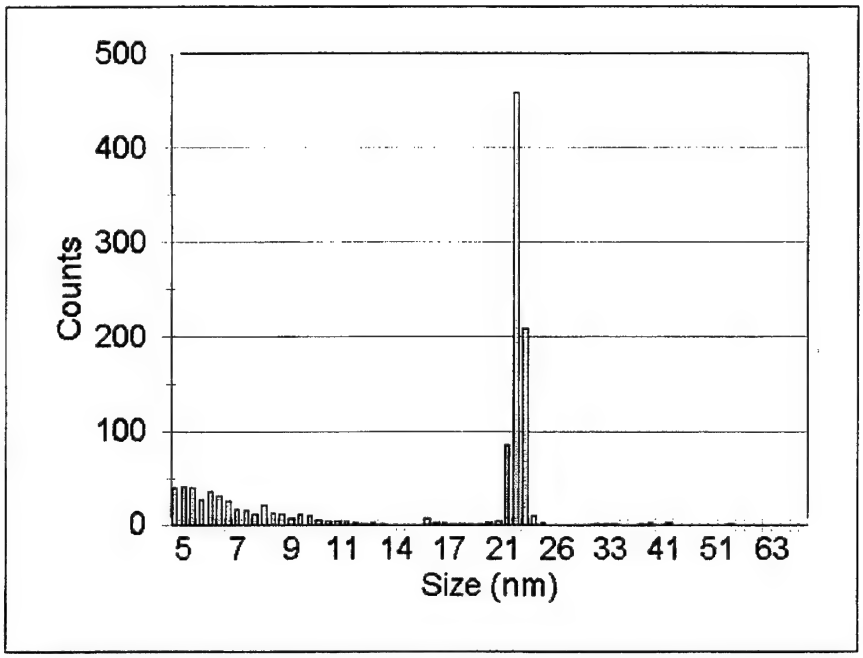

Figure 25 GEMMA Scan of $2x10^{11}$ pfu/ml MS2

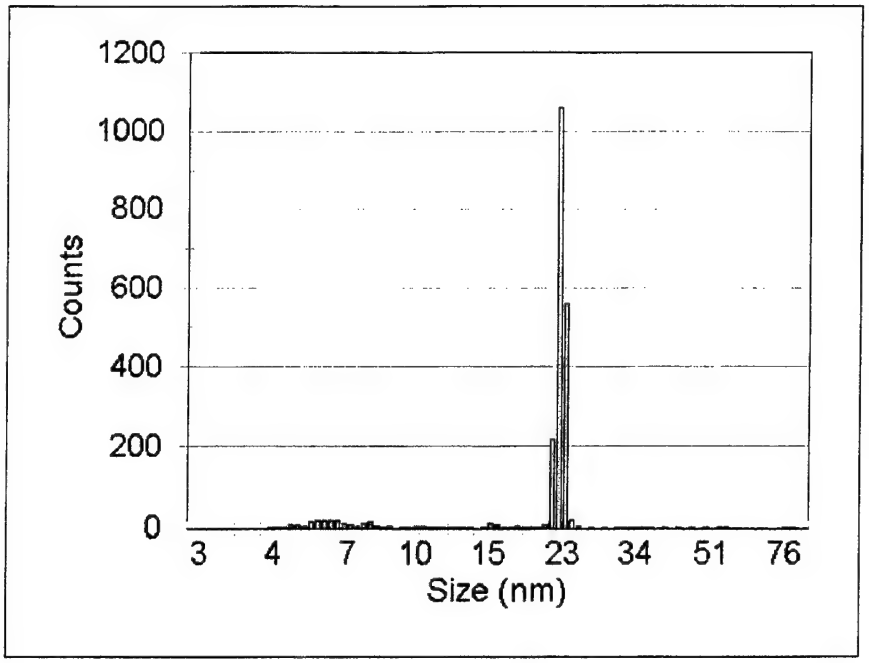

Figure 26 GEMMA Scan of $2x10^{11}$ pfu/ml MS2 after Application of 2nd Module

Processing 200 microliters of a $2x10^{10}$ pfu/ml MS2 solution through module 1 resulted in a sample of only $4x10^{9}$ pfu. This was injected into the 2nd module and, after application of four diafiltrations by the 2nd module, an excellent signal/noise ratio was obtained. The results of analyzing this sample are presented in Figure 27.

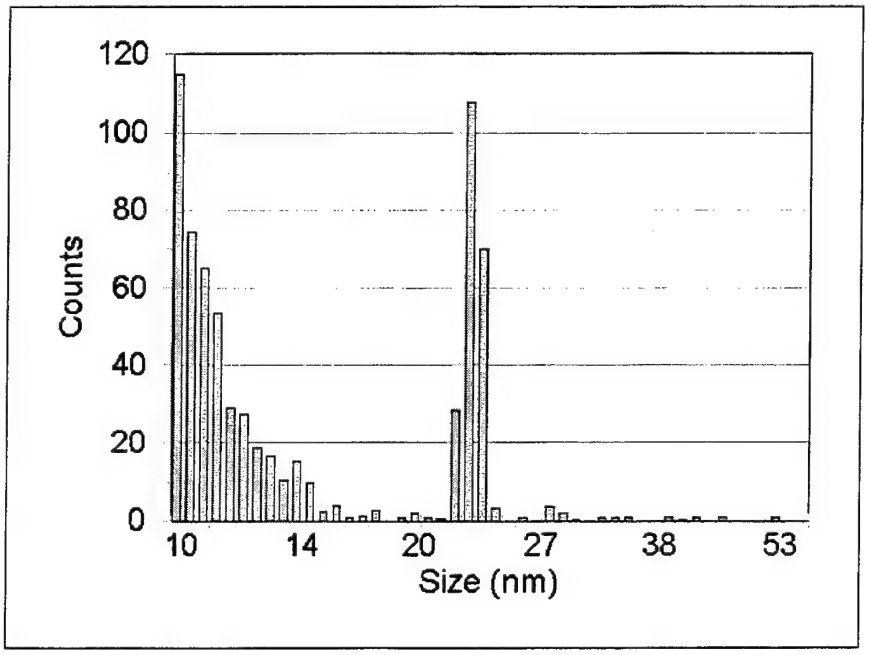

Figure 27 MS2 Aliquot ($4x10^{9}$ pfu) after Four Diafiltrations with 2nd Module

With a peak amplitude of over 100 counts/channel, the amplitude drops to a few counts per channel only three channels to either side of the maximum. This demonstrates a signal/noise ratio of about 25:1, with a size resolution of about ±1 nm. Furthermore, the fact that 4 diafiltrations were performed and 100 counts/channel were obtained at this input concentration means that the 2nd module is able to retain a high fraction of the viruses throughout 5 compression steps.

Processing 250 microliters of an $8x10^7$ pfu/ml MS2 solution through module 1 yielded a starting amount was only $2x10^7$ pfu. This sample was fed into the 2nd module and, after applying one compression, a very good signal/noise was obtained, as shown in Figure 28. The amplitude drops from 200 counts/channel in the peak area to 7 or fewer counts/channel five channels to either side of the highest peak channel, making the signal/noise ratio better than 25:1.

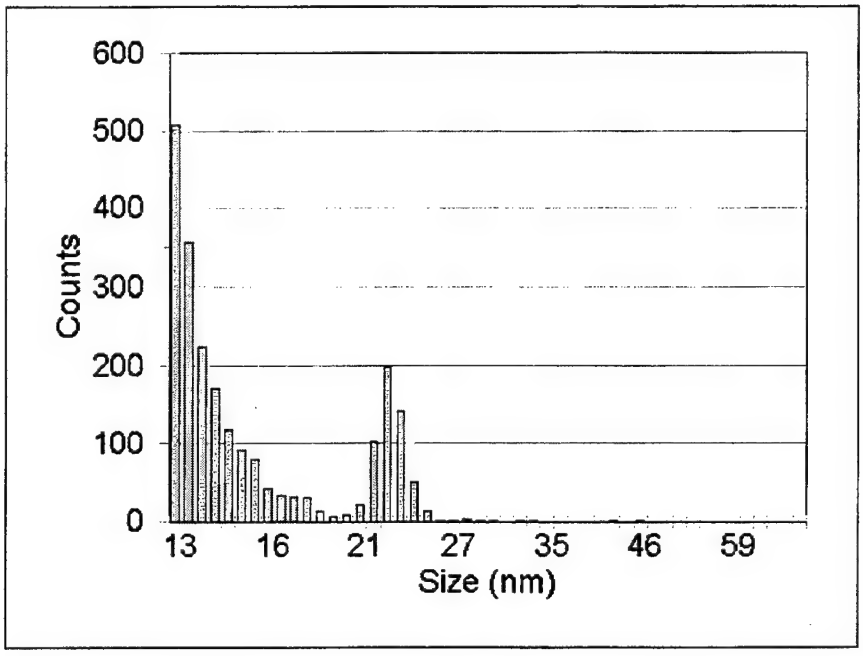

Figure 28 MS2 Aliquot ($2x10^7$ pfu) after One Diafiltration with 2nd Module

5.6.6 Ultrafiltration-GEMMA Tandem Analysis of MS2 Solutions with Thyroglobulin.

5.6.6.1 Results

A 1 wt% solution of the large protein thyroglobulin, with $5x10^{10}$ pfu of MS2 added, was processed through both the 1st and 2nd modules of the UF. Module 1 was equipped with a 750K Dalton cross flow filter cartridge and Module 2 was fitted with a 1M Dalton filter disk. The resulting GEMMA analysis, presented in Figure 29, shows a

virus peak, though with intensities somewhat lower than would be expected based on the previously discussed experiments. One would expect more on the order of 100 counts per channel in the peak. Due to the low count rate, it would appear likely that the 1st module is causing some loss or fragmentation of virus under the conditions used.

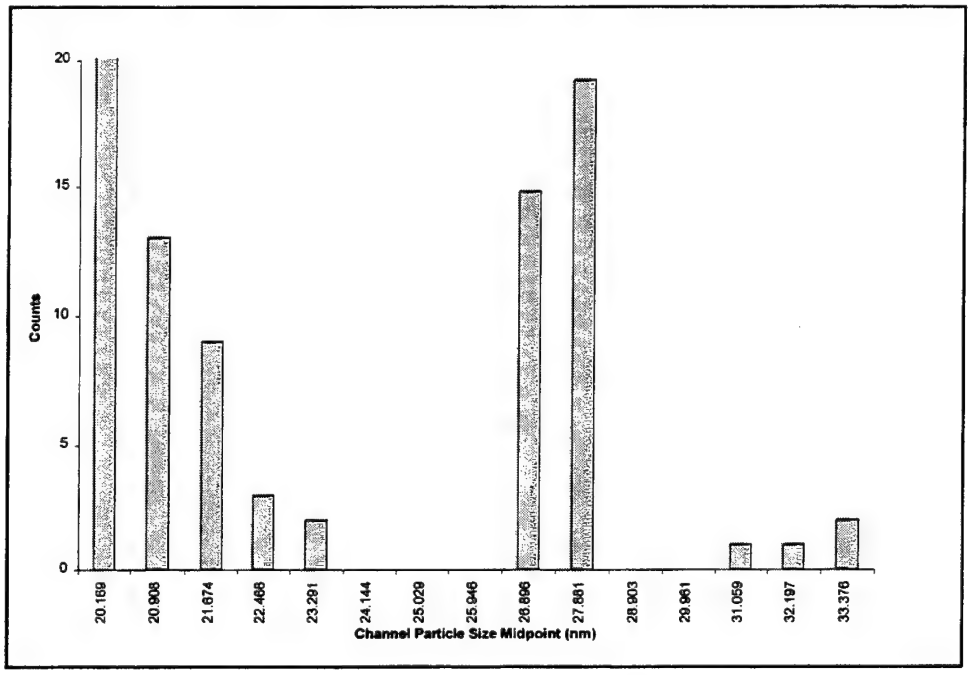

Figure 29 GEMMA Scan of 1% Thyroglobulin + $5x10^{10}$ pfu MS2 after Treatment in UF Modules 1 and 2

5.6.6.2 Analysis of Ultrafiltration-GEMMA Tandem Analysis of MS2 Solutions with Thyroglobulin

The removal of thyroglobulin by the first module is shown to be excellent in Figure 30. A 1% concentration of any soluble (and non-volatile) impurity should give rise to a broad "salt peak" centered at about 60 nm, whereas the salt peak in these data lies at 10 nm (and with a low intensity of less than 400 counts). A ten-fold reduction in the center position of the salt peak indicates (because of the third-power dependence of the volume of a sphere on the diameter) a thousand-fold reduction in the concentration of the impurity. Thus the UF has reduced the thyroglobulin concentration from 1% down to 0.001% or less. It should be mentioned that there is no known malfunction of the GEMMA that could conceivably give rise to a salt peak at only 10 nm size when the concentration of any soluble impurity is significantly greater than 0.001%.

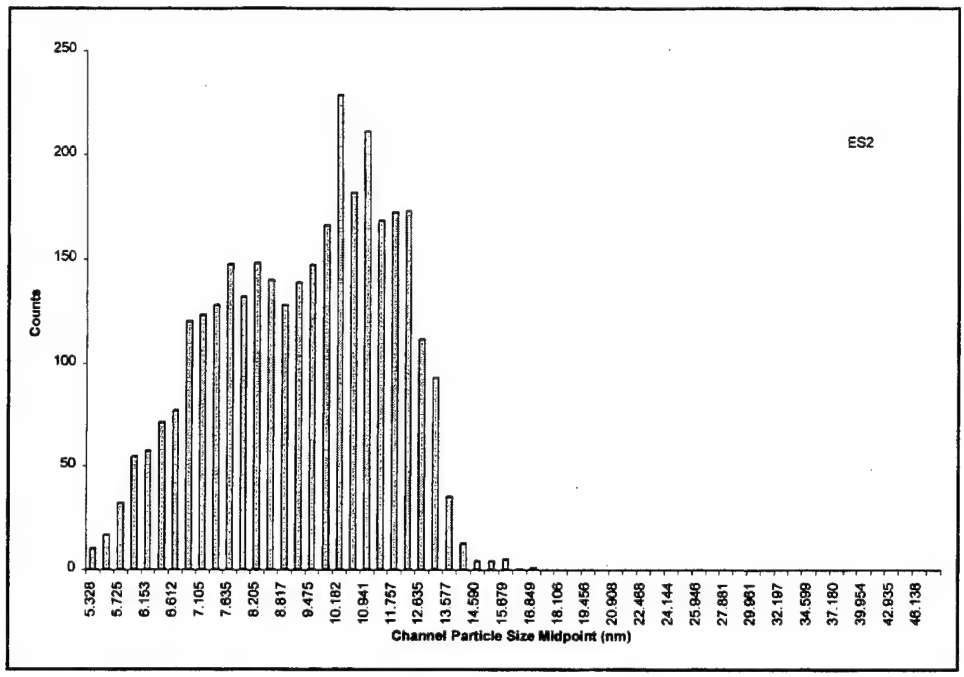

Figure 30 GEMMA Scan of 1% Thyroglobulin + $5x10^{10}$ pfu MS2 after Treatment in UF Modules 1 and 2

This 1,000-fold reduction of thyroglobulin concentration is quite remarkable, considering the following. First, the MW of the protein is only slightly smaller than the rated MW of the hollow fiber used in the 1st module, which was 750 kD. It should be mentioned that the company which supplies our 750 kD, low internal volume hollow fiber cartridge will soon be marketing a 1 million D cartridge, which will yield much better performance with large proteins such as this. Second, due to its non-TFF operation, the 2nd module of the UF is probably not adding a lot to the removal of this protein, so that most of this 1,000-fold reduction is coming from the 1st module. Third, the appearance of the virus peak as shown in Figure 29 demonstrates that at least a portion of the virus is being retained even as the large protein is being almost completely removed. Studies are ongoing to extend this type of result to a wider range of proteins.

5.7 Conclusions.

- MS2 bacteriophage, grown in a bacterial culture, and rich in biological impurities, was purified by density-gradient centrifugation, followed by dialysis (very closely related to ultrafiltration), in a purification protocol that simulates that envisioned for the final IVDS system. This methodology, in addition to purification, also effects a tremendous concentration of the virus.
- After purification and concentration with the gradient centrifugation and dialysis protocol, MS2 bacteriophage yields a huge GEMMA detector signal of 40,000 counts per channel and a signal-to-noise ratio better than 10,000:1.
- In addition to intact virus particles, there is evidence of viral fragments and viral RNA in the detector data, which yield at least semi-quantitative information on the degree of fragmentation of the virus. Furthermore, information on the degree of complexation of the virus was dramatic in that the peak corresponding to MS2 dimers was an order of magnitude larger than that for single virus particles, for freshly prepared MS2 samples. This dimerization broke down over a month in storage to yield a high fraction of monomeric MS2. The dimerization can be understood by reference to electron micrographs of MS2, from the literature, that show MS2, as prepared from bacterial cultures, arranged as pairs on the bacterial pili, like barbells.
- A solution of $2x10^8$ pfu/ml was detected with a small number of counts but with a dramatic signal/noise ratio. Indeed, nearly every channel outside the virus peak region registered zero counts.
- A large protein, thyroglobulin, at a concentration of 1% in a $2x10^9$ pfu/ml MS2 solution, was successfully removed by the UF and yielded a good signal/noise ratio, though there was some loss of virus due to the action of the 1^{st} module.
- Standardized particles of 74 nm and 54 nm diameter and known concentration were obtained and used to calibrate the GEMMA for both size and concentration measurements.
- Using standardized salt solutions, the linearity of the detector was determined (along with the electrospray droplet size), as well as the point at which linearity breaks down due to changing of the liquid conductivity.
- An E-Coli T7 bacteriophage was established as a second, standard virus for use in IVDS evaluations. Interestingly, our work indicates that a single pfu, as obtained from ATCC, may contain as many as 1,000 virus particles.

5.8 Recommendations for Additional Development and Testing.

There is a need to upgrade the CPC. A replacement CPC model 3010 will be available in the future that will increase the sensitivity by a factor of 30. There will always be a need for greater sensitivity in the GEMMA unit to count lower numbers of viruses in solution.

There is also a need to calibrate the GEMMA unit and ultrafiltration apparatus with many more known viruses. Each new virus has considerable information, which has not been fully characterized with these technologies. Size and shape and how the virus behaves in the GEMMA unit and the ultrafiltration apparatus needs to be examined. The examination of other viruses will also determine if there are any other factors that require further processing that differs from already examined samples.

The further analysis of differing viruses will facilitate the generation of a database to automatically determine the pedigree of a particular particle size peak. When there is a sufficient variety of viruses analyzed, it will be easy to generate the software to determine what virus families a particular GEMMA scan is displaying. This information could then be displayed in an easily interpreted format.

Ultracentrifugation is a technique where virus particles are physically separated from other biological material. When viruses are centrifuged in a salt solution, the various families of viruses separate into density gradients. By capturing specific density slices from the output of a zonal centrifuge and then determining the size distributions of the viruses in solution, the specific virus families present can be identified. This technology needs to be incorporated into the flow of sample preparation protocols for virus analysis. An interesting article[7] pertaining to the incorporation of ultracentrifugation into the IVDS shows that the new nanotechnology revolution may offer a solution to the miniaturization of a zonal centrifuge apparatus. The realization of a nanocentrifuge, only hundreds of nanometers in size, could be directly incorporated into the existing hardware of the GEMMA detector and the ultrafiltration apparatus.

[7] Nanomedicine, (Ch. 3.2.5) by Robert A. Freitas Jr. to be published by Landes Biosciences (Spring 1999), see link:http://foresight.org/Nanomedicine/Ch03_1.html

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GLOSSARY

Bacteriophage – a virus that attacks bacteria
Cross flow – sweeping action created by fluid flow across a membrane
CPC – condensate particle counter
Dalton (D) – molecular weight of macromolecules
Diafiltration – dialysis type of filtration
Dialysis – the separation of salts and microsolutes from macromolecular solutions by a concentration gradient
DMA – differential mobility analyzer
Electrospray (ES) – the process of atomizing a liquid by injecting across an electrical potential
GEMMA - Gas-phase Electrophoretic Mobility Molecular Analyzer
IVDS – Integrated Virus Detection System
MW – molecular weight
Phage – see bacteriophage
Plaque forming unit (pfu) – quantity of a virus required to form a viable colony
MS2 – type of bacteriophage
MWCO – molecular weight cut-off
SMPS - Scanning Mobility Particle Sizer
T7 - type of bacteriophage
Tangential flow – see cross flow
Ultrafiltration (UF) - the separation of salts and microsolutes from macromolecular solutions by hydrostatic pressure